

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>A01N 37/18, A61K 38/00, 38/04, G01N 33/53, 33/574, 33/48</b>	A1	(11) International Publication Number: <b>WO 98/58541</b> (43) International Publication Date: 30 December 1998 (30.12.98)
(21) International Application Number: <b>PCT/US98/12595</b>		(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: <b>16 June 1998 (16.06.98)</b>		
(30) Priority Data: <b>08/881,646 24 June 1997 (24.06.97) US</b>		<b>Published</b> <i>With international search report.</i>
(71) Applicants: DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US). NOVARTIS AG [CH/CH]; CH-4002 Basel (CH).		
(72) Inventors: KUFE, Donald, W.; 179 Grove Street, Wellesley, MA 02181 (US). KHARBANDA, Surender, M.; 13 Bradford Road, Natick, MA 01760 (US). NALIN, Carlo, M.; 327 Forrest Glen Avenue, Franklin Lakes, NJ 07417 (US). SHARMA, Sushil, K.; 15 Locust Street, Budd Lake, NJ 07828 (US).		
(74) Agent: FRASER, Janis, K.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02115 (US).		

(54) Title: MODULATION OF APOPTOSIS

## (57) Abstract

Peptides and peptidomimetics that inhibit binding of cytochrome C to a Bcl-2-related family member, for use in therapy of autoimmune diseases and cancer as well as in prevention of graft rejection in organ transplant patients, by administering either the peptide, its peptidomimetic or an expression vector encoding the peptide.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

## MODULATION OF APOPTOSIS

5        This invention was made in part with government support under grant number CA66996 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

### Background of the Invention

10      The invention relates to compounds and methods useful in regulating apoptosis.

Total cell numbers in higher organisms (eukaryotes) are regulated at the levels of both cell division and cell death. An active mechanism of 15 programmed cell death known as apoptosis has been described, whereby large numbers of cells die during normal development and tissue homeostasis [Kerr et al. (1972) Br. J Cancer 26:239]. The cascade of intrinsic biochemical reactions resulting in the eventual death of 20 the relevant cell may be initiated by external or internal stimuli. Features of apoptosis include cell membrane "blebbing", cell shrinkage, nuclear condensation and degradation of DNA at nucleosomal intervals [Wyllie et al. (1980) Int. Rev. Cytol. 68:251].

25      The cellular response to ionizing radiation (IR) and other genotoxic agents includes cell cycle arrest and activation of DNA repair. In the event of irreparable DNA damage, cells respond with induction of apoptosis. However, the intracellular signals that control the 30 induction of apoptosis are unclear. Whereas p53 has been implicated in promoting apoptosis induced by IR exposure [Lowe et al. (1993) Nature 362:847; Clarke et al. (1993) Nature 362:849], other studies have demonstrated that the

- 2 -

Bcl-2 and Bcl-x<sub>L</sub> proteins inhibit IR-induced apoptosis [Sentman et al. (1991) Cell 67:879; Strasser et al. (1991) Cell 67:889; Datta et al. (1995) Cell Growth and Differ. 6:363]. Members of the anti-apoptotic Bcl-2-related family of proteins are expressed in the mitochondrial membrane [Monaghan et al. (1992) J. Histochem. Cytochem. 40:1819; Gonzalez-Garcia et al. (1994) Development 120:3033]. The crystal structure of Bcl-x<sub>L</sub> supports an arrangement of  $\alpha$ -helices that is found in the membrane translocation domains of bacterial toxins [Muchmore et al. (1996) Nature 381:335; Parker and Pattus (1993) Trends Biochem. Sci. 18:391].

The induction of apoptosis by diverse stimuli, including IR, is associated with activation of aspartate-specific cysteine proteases (caspases) [Martin and Green (1995) Cell 82:349] and cleavage of poly(ADP-ribose) polymerase (PARP) [Kaufman et al. (1993) Cancer Res. 53:3976], protein kinase C  $\delta$  (PKC $\delta$ ) [Emoto et al. (1995) EMBO J. 14:6148], and other proteins. The finding that cleavage of PARP and PKC $\delta$  is blocked by Bcl-x<sub>L</sub> has suggested that the Bcl-2-related family of anti-apoptotic proteins functions upstream of the activation of caspases [Emoto et al. cited supra; Chinnaiyan et al. (1996) J. Biol. Chem. 271:4573; Datta et al. (1997) J. Biol. Chem. 272:1965]. More direct evidence for the involvement of caspases in apoptosis is derived from studies with the baculovirus protein p35 which directly inhibits cysteine proteases and blocks induction of apoptosis [Datta et al. (1997), cited supra; Bump et al. (1995) Science 269:1885].

Recent work has suggested that mitochondria play a role in inducing apoptosis by releasing cytochrome C [Liu et al. (1996) Cell 83:147]. Addition of cytochrome C and dATP to cytosolic preparations from growing cells activates caspases, such as CPP32 [Liu et al. cited supra], responsible for cleavage of PARP and PKC $\delta$  [Tewari

- 3 -

et al. (1995) Cell 81:801; Nicholson et al. (1995) Nature 376:37; Ghayur et al. (1996) J. Exp. Med. 184:2399]. Cytochrome C also induces DNA fragmentation in isolated nuclei incubated with cytosolic lysates [Liu et al. cited 5 supra]. The finding that intact cells undergo apoptosis following release of cytochrome C into the cytosol has provided further support for an apoptotic function of this mitochondrial protein [Liu et al. cited supra].

Summary of the Invention

10 The invention is based on the discovery that Bcl-x<sub>L</sub> blocks release of cytochrome C into cellular cytosol by binding to cytochrome C, thereby inhibiting apoptosis. Cytochrome C has been found to bind directly to the anti-apoptotic, full-length Bcl-x<sub>L</sub>, and not the pro-apoptotic 15 alternatively spliced form of Bcl-x (designated Bcl-x<sub>S</sub>). Furthermore, certain peptide fragments of the region of Bclx<sub>L</sub> that is deleted in Bcl-x<sub>S</sub> block this binding.

The invention features a peptide or peptidomimetic that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family. The anti-apoptotic member of the Bcl-2-related family is preferably Bcl-x<sub>L</sub> or Bcl-2. The peptide may be a fragment of a Bcl-2-related family member and is preferably three to seventy amino acids long, is more preferably a 25 fragment of Bcl-x<sub>L</sub> or Bcl-2, even more preferably contains the amino acid sequence Ala-Leu-Cys-Val-Glu (SEQ ID NO:3) or Val-Met-Cys-Val-Glu (SEQ ID NO:4), and most preferably includes the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

30 Also included in the invention is a peptide that is five to seventy amino acids long and contains the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4.

The invention encompasses methods for screening for compounds that promote or inhibit apoptosis. In one 35 aspect, the screening methods involve: (a) providing

- 4 -

cytochrome C (or a derivative of cytochrome C such as apo-cytochrome C or porphyrin-cytochrome C) bound to a solid support; (b) contacting the cytochrome C (or a derivative of cytochrome C such as apo-cytochrome C or 5 porphyrin-cytochrome C) with a polypeptide comprising the sequence of SEQ ID NO:3 or SEQ ID NO:4 or a peptide that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family, in the presence of a test compound; and (c) determining whether the test 10 compound inhibits or enhances binding of the polypeptide or peptide to cytochrome C (or a derivative of cytochrome C such as apo-cytochrome C or porphyrin-cytochrome C). In a second aspect, the screening methods involve: (a) providing a polypeptide comprising the sequence of SEQ ID 15 NO:3 or SEQ ID NO:4 or a peptide that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family, the polypeptide or peptide being bound to a solid support; (b) contacting the polypeptide or peptide with cytochrome C (or a derivative of cytochrome 20 C such as apo-cytochrome C or porphyrin-cytochrome C) in the presence of a test compound; and (c) determining whether the test compound inhibits or enhances binding of cytochrome C (or a derivative of cytochrome C such as apo-cytochrome C or porphyrin-cytochrome C) to the 25 polypeptide or peptide. In both these screening methods, inhibition of binding is an indication that the test compound promotes apoptosis and enhancement of binding is an indication that the test compound inhibits apoptosis.

In both these screening methods the polypeptide 30 containing the sequence of SEQ ID NO:3 or SEQ ID NO:4 can be (a) Bcl-x<sub>L</sub>; (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy-terminus of naturally occurring Bcl-x<sub>L</sub>; (c) Bcl-2; (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy 35 terminus of naturally occurring Bcl-2; or (e) a fusion

- 5 -

protein containing any one of (a) - (d). Truncation at the carboxy terminus increases solubility of these proteins.

Another polypeptide comprising the sequence of SEQ ID NO:3 or SEQ ID NO:4 and which may also be used in both screening methods is a peptide that is five to seventy amino acids long and is preferably a fragment of Bcl-x<sub>L</sub> or Bcl-2.

Another embodiment of the invention is an antibody which binds specifically to an epitope within the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4. The antibody is preferably a monoclonal antibody.

The invention features an expression vector comprising a polynucleotide that encodes a peptide that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family. Also within the invention is a method for inhibiting binding of cytochrome C to Bcl-x<sub>L</sub>. The method includes the steps of introducing into a cell a peptide or a peptidomimetic that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family. The cell is preferably in a mammal such as a human, non-human primate, mouse, rat, rabbit, guinea pig, hamster, cow, horse, sheep, goat, dog or cat. The peptide or its peptidomimetic may be introduced into the cell of the mammal by directly administering the peptide or peptidomimetic to the mammal. The peptide may alternatively be introduced to the cell of the mammal by administering an expression vector containing a polynucleotide that encodes the peptide. The mammal is one having a condition wherein apoptosis in at least one cell type is desirable for the health and homeostasis of the individual, for example, an individual having an autoimmune disease or cancer, or a transplant recipient. Where the patient has cancer, the method may be carried

- 6 -

out in conjunction with chemotherapy, radiation therapy or immunotherapy.

The invention includes a nucleic acid that encodes a polypeptide containing multiple peptide domains, each 5 of which includes the amino acid sequence of SEQ ID NO:3. A method for inhibiting release of cytochrome C into cell cytosol is also a component of the invention. The method may involve introducing into appropriate cells of a patient (e.g., a human suffering from an 10 immunodeficiency disease) the nucleic acid that encodes a polypeptide comprising multiple peptide domains, each of which contains the amino acid sequence of SEQ ID NO:3. The nucleic acid can be introduced into the patient's cells by administering the nucleic acid to the patient.

15 By "Bcl-2-related family" is meant the group of homologous polypeptides that includes Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1, Bax, Bak, Bcl-x<sub>S</sub>, Bad, Bid, Bik and Hrk [Kroemer (1997) *Nature Medicine* 3:614].

By "Bcl-x<sub>L</sub>" is meant the full-length, mature Bcl-20 x<sub>L</sub> polypeptide [Boise et al. (1993) *Cell* 74:597].

By "Bcl-x<sub>S</sub>" is meant the full-length, mature Bcl-x<sub>S</sub> polypeptide [Boise et al., cited supra].

By "Bcl-2" is meant the full length, mature Bcl-2 polypeptide [Cleary et al. (1986) *Cell* 47:19].

25 By "peptide" is meant a polypeptide of at least 2 amino acids and not more than one hundred amino acids in length.

By "encodes" a given peptide is meant that the coding sequence consists solely of codons defining the 30 amino acid sequence of the given peptide.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and 35 materials similar or equivalent to those described herein

- 7 -

can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples described herein are illustrative only and not intended to be limiting.

Other features and advantages of the invention, e.g., therapy for a variety of human diseases, will be apparent from the following detailed description, from the drawings and from the claims.

Brief Description of the Drawings

Fig. 1A is a line graph illustrating the binding of Gst-Bcl-x<sub>LT</sub> to cytochrome C in an ELISA.

Fig. 1B is a bar graph illustrating the inhibition of binding of GST-Bcl-x<sub>LT</sub> to cytochrome C by GST-Bcl-x<sub>ST</sub> in an ELISA.

Fig. 2 is a diagram depicting linear representations of Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub> polypeptides. The region of the Bcl-x<sub>L</sub> molecule not present in the Bcl-x<sub>S</sub> molecule, designated  $\Delta X_{L/S}$ , is also shown. Below the  $\Delta X_{L/S}$  structure is illustrated the panel of decamer peptides that were synthesized and are described in greater detail in Fig. 3.

Fig. 3 is a diagram depicting the  $\Delta X_{L/S}$  region which spans amino acids 126 to 189 of the Bcl-x<sub>L</sub> molecule. The diagram also illustrates a panel of twelve overlapping decamer peptides which together span the whole  $\Delta X_{L/S}$  region. The peptides are appropriately aligned relative to the  $\Delta X_{L/S}$  region and the numbers assigned to each peptide are shown. All the peptides except peptide (12) contain a 5 amino acid overlap with

- 8 -

the peptide immediately preceding it (see also Table 1). Peptide (12) contains seven amino acids of peptide (11). The amino acid sequences of peptide (7) (SEQ ID NO:1) and peptide (8) (SEQ ID NO:2) are given, as is the amino acid 5 sequence of their region of overlap (SEQ ID NO:3).

Fig. 4 is a line graph illustrating the inhibition of binding of Bcl-x<sub>L</sub> to cytochrome C by ΔX<sub>L/s</sub> peptides (7) (SEQ ID NO:1) and (8) (SEQ ID NO:2) in an ELISA.

#### Detailed Description

10

##### A. Overview

When cells are exposed to apoptosis-inducing agents, cytochrome C is released from mitochondria into the cell cytosol. This released cytochrome C initiates the cascade of biochemical reactions that culminate in 15 apoptosis of the cell. It has now been discovered that Bcl-x<sub>L</sub> inhibits both cytochrome C release into the cytosol and the sequelae that lead to apoptosis of the cell. Bcl-x<sub>L</sub> inhibits cytochrome C release, and hence also apoptosis, by binding to it and thereby preventing it 20 from exiting mitochondria and entering the cytosol. Also of great interest is the observation that certain peptide fragments of Bcl-x<sub>L</sub> inhibit binding of Bcl-x<sub>L</sub> to cytochrome C. These and other peptides (or peptidomimetics) that inhibit binding of Bcl-2-related 25 family members to cytochrome C, expression vectors encoding such peptides, antibodies that specifically bind to them and therapeutic or screening methods utilizing them are aspects of the invention.

Western blot analysis showed that U-937 human 30 myeloid leukemia cells exposed to 20 Gy of ionizing radiation, cisplatin (CDDP) (50 μm) or methyl methanesulfonate MMS (1mM), responded with (a) increased levels of cytosolic cytochrome C; and (b) cleavage of PARP into characteristic apoptotic fragments.

- 9 -

Furthermore, after exposure to IR, mitochondrial cytochrome C was depleted.

In U-937 cells overexpressing a gene that encodes Bcl-x<sub>L</sub> (U-937/Bcl-x<sub>L</sub>), accumulation of cytosolic cytochrome C in response to IR or CDDP treatment was prevented. In addition, treatment of U-937/Bcl-x<sub>L</sub> cells with IR or CDDP did not result in PARP cleavage. To address the possibility that cytochrome C may function upstream of the activation of cysteine proteases, U-937 cells stably overexpressing the cysteine protease inhibitor p35 were irradiated and their cytosolic proteins analyzed. This experiment showed that p35 had no detectable effect on accumulation of cytosolic cytochrome C, but blocked cleavage of PARP and internucleosomal DNA fragmentation. From this result it was concluded that cytochrome C release from mitochondria in response to apoptotic stimuli is indeed upstream of the activation of cysteine proteases. Since overexpression of Bcl-x<sub>L</sub> blocked the increase in cytosolic cytochrome C, co-immunoprecipitation studies were performed in order to determine whether the two proteins form a specific complex in cells. Lysates from control unirradiated and irradiated U-937/Bcl-x<sub>L</sub> cells were subjected to immunoprecipitation with antibody specific for cytochrome C, and the resulting immunoprecipitates were analyzed with antibody specific for Bcl-x (i.e., both Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>). The data showed that the cytochrome C-specific antibody co-precipitated Bcl-x<sub>L</sub> but not Bcl-x<sub>S</sub>, whether or not the cells had been irradiated. This finding was the first to demonstrate a physical association between a Bcl-2-related family member and cytochrome C.

To obtain further evidence of this intermolecular association, a recombinant truncated fusion protein consisting of Bcl-x<sub>L</sub> lacking 21 carboxy-terminal acids of

- 10 -

naturally occurring Bcl-x<sub>L</sub> (designated Bcl-x<sub>LT</sub>) and fused to hexahistidine (H6) was resolved by gel electrophoresis, transferred to a nitrocellulose filter and renatured in aqueous buffer. Consecutive exposure to 5 cytochrome C and cytochrome C-specific antibody demonstrated a direct interaction of cytochrome C and Bcl-x<sub>L</sub>, but no such interaction between cytochrome C and Bcl-x<sub>ST</sub> (recombinant, truncated Bcl-x<sub>S</sub> lacking 21 carboxy-terminal amino acids of natural Bcl-x<sub>S</sub>) or a control 10 protein, MAP kinase (MAPK). These results showed that the binding of cytochrome C to Bcl-x<sub>L</sub> is specific.

In a converse experiment, purified cytochrome C was resolved by electrophoresis and transferred to nitrocellulose filters, which were incubated with 15 purified H6, H6-Bcl-x<sub>LT</sub> or Bcl-x<sub>ST</sub> and analyzed by immunoblotting with anti-Bcl-x antibody. Only the filters exposed to H6-Bcl-x<sub>LT</sub> showed reactivity with cytochrome C, thereby again demonstrating a specific interaction between cytochrome C and Bcl-x<sub>L</sub>. This model 20 was supported by an ELISA in which cytochrome C adsorbed to wells of a plastic ELISA plate was incubated with glutathione-S-transferase (GST) (as a control), or a fusion protein of GST and either Bcl-x<sub>LT</sub> (GST-Bcl-x<sub>LT</sub>) or Bcl-x<sub>ST</sub> (GST-Bcl-x<sub>ST</sub>). Unbound proteins were removed by 25 washing. Rabbit antibody specific for GST, enzyme-conjugated anti-rabbit Ig (immunoglobulin) and a fluorogenic substrate for the enzyme were consecutively added to the wells of the ELISA plate. (Intermediate washing steps were included.) The results showed that 30 GST-Bcl-x<sub>LT</sub>, but not GST or GST-Bcl-x<sub>ST</sub>, bound to the adsorbed cytochrome C.

While Bcl-x<sub>S</sub> itself failed to bind to cytochrome C, an ELISA indicated that it inhibited binding of Bcl-x<sub>L</sub> to cytochrome C. These ELISA experiments confirmed that 35 the binding between cytochrome C and Bcl-x<sub>L</sub> shown by co-

- 11 -

immunoprecipitation of cellular lysates is due to a specific interaction which can be blocked by Bcl-x<sub>s</sub>. Bcl-x<sub>s</sub> is known to form a heteroduplex with Bcl-x<sub>L</sub> [Sato et al. (1994) Proc. Natl. Acad. Sci. USA 91:9238]

5 These studies demonstrate that, by binding to cytochrome C, Bcl-x<sub>L</sub> blocks the increase in cytosolic cytochrome C accumulation associated with induction of apoptosis by IR and genotoxic drugs. This interaction is specific for Bcl-x<sub>L</sub> and not the pro-apoptotic Bcl-x<sub>s</sub>

10 protein. These findings support a model in which release of cytochrome C from mitochondria during induction of apoptosis can be prevented by binding of cytochrome C to Bcl-x<sub>L</sub>, thereby decreasing apoptosis. Conversely, inhibiting the binding of cytochrome C to Bcl-x<sub>L</sub> will

15 permit release of cytochrome C into the cytosol, triggering the downstream events that lead to apoptosis.

The above experiments show that Bcl-x<sub>L</sub> binds to cytochrome C and Bcl-x<sub>s</sub> does not bind to cytochrome C. Bcl-x<sub>s</sub> lacks an internal 63 amino acid domain ( $\Delta X_{L/S}$ ) of 20 Bcl-x<sub>L</sub> [Boise et al., cited supra]. Therefore, the possibility that residues within  $\Delta X_{L/S}$  are involved in binding of Bcl-x<sub>L</sub> to cytochrome C was tested. A panel of twelve overlapping decamer peptides spanning  $\Delta X_{L/S}$  were synthesized for use in binding inhibition experiments.

25 These peptides were tested for their ability to inhibit binding of Bcl-x<sub>L</sub> to cytochrome C in an ELISA similar to that described for inhibition of binding to chromosome C of Bcl-x<sub>L</sub> by Bcl-x<sub>s</sub> (see supra). Inhibition was measured at a range of peptide concentrations (1-100  $\mu$ M). Peptide 30 (7) (SEQ ID NO:1) and peptide (8) (SEQ ID NO:2) decreased binding of Bcl-x<sub>L</sub> to cytochrome C to a level approximately 20% of the control without peptide (i.e. 80% inhibition). The carboxy-terminus of peptide (8) (Phe-Ser-Phe-Gly-Gly-Ala-Leu-Cys-Val-Glu) (SEQ ID NO:2) overlaps the amino- 35 terminus of peptide (7) (Ala-Leu-Cys-Val-Glu-Ser-Val-Asp-

- 12 -

Lys-Glu) (SEQ ID NO:1) by 5 amino acids. The amino acid sequence of this region of overlap is Ala-Leu-Cys-Val-Glu (SEQ ID NO:3). A pentameric peptide with this sequence inhibited binding of Bcl-x<sub>L</sub> to cytochrome C almost as well  
5 (approximately 3-fold less avidly) as peptide (7) (SEQ ID NO:1) or peptide (8) (SEQ ID NO:8).

Alignment of Bcl-x<sub>L</sub> and Bcl-2 molecules reveals that the region of Bcl-2 equivalent to SEQ ID NO:3 has the amino acid sequence Val-Met-Cys-Val-Glu (SEQ ID  
10 NO:4). In light of the similarity between SEQ ID NO:3 and SEQ ID NO:4 and the conservative nature of the two amino acid difference (Ala-Leu in SEQ ID NO:3 versus Val-Met in SEQ ID NO:4), it is presumed that Bcl-2 also regulates cytochrome C release from mitochondria [Yang et  
15 al. (1997) *Science* 275:1129; Kluck et al. (1997) *Science* 275:1132] by binding to cytochrome C, and that it does so via an interaction involving all or a portion of the five amino acid domain of SEQ ID NO:4. Peptides containing  
the amino acid sequences of either or both of SEQ ID NO:3  
20 and SEQ ID NO:4 may be used in the methods of invention.

As described infra, the peptides and nucleic acids encoding them can be used for therapy of a variety of disease conditions, including human diseases. The peptides can also be used to generate and screen for  
25 antibodies specific for epitopes within the amino acid sequence of SEQ ID NOS:1, 2, 3 or 4. The resulting antibodies could be used in screening assays analogous to those described infra. Such antibodies are another aspect of the invention.

30 While the results of the binding inhibition experiments indicate that peptides (7) (SEQ ID NO:1) and (8) (SEQ ID NO:2) define a dominant site on Bcl-x<sub>L</sub> for binding of cytochrome C, they also suggest that this site is not the only site involved in binding. The latter  
35 possibility is shown by the fact that peptides other than

- 13 -

(7) (SEQ ID NO:1) and (8) (SEQ ID NO:2) showed less but still significant ability to inhibit binding of Bcl-x<sub>L</sub> to cytochrome C. It is likely that such peptides would show at least additive inhibition of binding of Bcl-x<sub>L</sub> to  
5 cytochrome C when used singly or multiply in combination with peptides (7) (SEQ ID NO:1) and/or (8) (SEQ ID NO:2) or others comprising SEQ ID NO:3 or SEQ ID NO:4. If the peptides mediate their inhibitory activity by inducing conformational changes as well as by a cognate  
10 competitive mechanism, the effects of combining peptides might also be synergistic. Such synergistic or additive properties might be advantageous for therapeutic methods (see infra). The sequences and SEQ ID NOS. of all the peptides used for the binding inhibition experiments are  
15 shown in Table 1.

Table 1. Peptides used in assays for inhibition of binding of GST-Bcl-x<sub>L</sub> to cytochrome C.

Peptide Number	Amino Acid Sequence	SEQ ID NO:
5 1	Glu-Pro-Trp-Ile-Gln-Glu-Asn-Gly-Gly-Trp	SEQ ID NO:5
2	Leu-Asn-Asp-His-Leu-Glu-Pro-Trp-Ile-Gln	SEQ ID NO:6
3	Trp-Met-Ala-Thr-Tyr-Leu-Asn-Asp-His-Leu	SEQ ID NO:7
4	Ser-Arg-Ile-Ala-Ala-Trp-Met-Ala-Thr-Tyr	SEQ ID NO:8
5	Met-Gln-Val-Leu-Ser-Arg-Ile-Ala-Ala	SEQ ID NO:9
10 6	Ser-Val-Asp-Lys-Glu-Met-Gln-Val-Leu-Val	SEQ ID NO:10
7	Ala-Leu-Cys-Val-Glu-Ser-Val-Asp-Lys-Glu	SEQ ID NO:1
8	Phe-Ser-Phe-Gly-Ala-Leu-Cys-Val-Glu	SEQ ID NO:2
9	Arg-Ile-Val-Ala-Phe-Phe-Ser-Phe-Gly-Gly	SEQ ID NO:11
10	Gly-Val-Asn-Trp-Gly-Arg-Ile-Val-Ala-Phe	SEQ ID NO:12
15 11	Glu-Leu-Phe-Arg-Asp-Gly-Val-Trp-Gly	SEQ ID NO:13
12	Val-Val-Asn-Glu-Leu-Phe-Arg-Asp-Gly-Val	SEQ ID NO:14

- 15 -

## B. PEPTIDES

### B.1 Peptide production

Peptides of the invention can be chemically synthesized by techniques familiar to those skilled in the art [e.g. see Creighton (1983) *Proteins: Structures and Molecular Principles*, W.H. Freeman and Co., N.Y.]. Larger peptides (longer than 50 amino acids) can be produced by a number of methods including recombinant DNA technology (see infra).

### 10 B.2 Peptide description

A peptide of the invention is a polypeptide of 2 to 100 residues that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family, such as Bcl-x<sub>L</sub>, Bcl-2, Bcl-w, Bfl-1, Brag-1, Mcl-1 or A1. The peptide may be a fragment of a Bcl-2-related family member. In a preferred embodiment the peptide is no more than 70 amino acids long and more preferably no more than 50 (e.g., less than 40 or even 30). Examples of such peptides would be three, four, five, six, seven, eight, nine, ten, twelve, fifteen, twenty or twenty-five amino acids in length. These peptides may be fragments of Bcl-x<sub>L</sub> or Bcl-2, and can contain the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4. Examples include peptides with the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or peptides containing such a sequence.

Another embodiment of the invention is a peptide three to seventy amino acids long containing at least three (e.g., Cys-Val-Glu) and preferably four residues of SEQ ID NO:3 or SEQ ID NO:4.

30 Peptides may also include variants of the peptides described supra. These variants would contain different amino acids (preferably conservative changes) from the

- 16 -

parental peptides but retain the ability to inhibit binding of cytochrome C to Bcl-2-related family members. Such variants can be synthesized by standard means, and are readily tested in the binding assays described above and 5 below.

Peptides of the invention will also include those described above but modified for *in vivo* use by:

(a) chemical or recombinant DNA methods to include mammalian signal peptides [Lin et al. (1995) J. Biol. Chem. 10 270:14255] or the bacterial peptide, penetratin [Joliot et al. (1991) Proc. Natl. Acad. Sci. USA 88:1864], that will serve to direct the peptide across cell and cytoplasmic membranes and/or traffic it to the mitochondrion;

(b) addition of a biotin residue which serves to 15 direct the peptide across cell membranes by virtue of its ability to bind specifically to a translocator present on the surface of cells [Chen et al. (1995) Analytical Biochem. 227:168];

(c) addition at either or both the amino- and 20 carboxy-terminal ends, of a blocking agent in order to facilitate survival of the relevant peptide *in vivo*. This can be useful in those situations in which the peptide termini tend to be degraded ("nibbled") by proteases prior to cellular or mitochondrial uptake. Such blocking agents 25 can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxy terminal residues of the peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology 30 (see Section C.1 infra). Alternatively, blocking agents such as pyroglutamic acid or other molecules known to those of average skill in the art may be attached to the amino and/or carboxy terminal residues, or the amino group at the

- 17 -

amino terminus or carboxyl group at the carboxy terminus replaced with a different moiety. Likewise, the peptides can be covalently or noncovalently coupled to pharmaceutically acceptable "carrier" proteins prior to administration.

5       Also of interest are peptidomimetic compounds based upon the amino acid sequence of the peptides of the invention. Peptidomimetic compounds are synthetic compounds having a three-dimensional structure (i.e. a "peptide motif") based upon the three-dimensional structure of a  
10 selected peptide. The peptide motif provides the peptidomimetic compound with the activity of inhibiting or enhancing the binding of cytochrome C to a member of the Bcl-2-related family that is the same or greater than the activity of the peptide from which the peptidomimetic was  
15 derived. Peptidomimetic compounds can have additional characteristics that enhance their therapeutic application such as increased cell permeability, greater affinity and/or avidity and prolonged biological half-life. The peptidomimetics of the invention typically have a backbone  
20 that is partially or completely non-peptide, but with side groups identical to the side groups of the amino acid residues that occur in the peptide on which the peptidomimetic is based. Several types of chemical bonds, e.g. ester, thioester, thioamide, retroamide, reduced  
25 carbonyl, dimethylene and ketomethylene bonds, are known in the art to be generally useful substitutes for peptide bonds in the construction of protease-resistant peptidomimetics.

**C. IN VITRO SCREENING ASSAYS FOR COMPOUNDS  
THAT INHIBIT OR ENHANCE APOPTOSIS**

30       *In vitro* systems may be designed to identify compounds capable of inhibiting or enhancing binding of cytochrome C to:

- 18 -

- (a) Bcl-x<sub>L</sub>, Bcl-2 or fusion proteins including Bcl-x<sub>L</sub> or Bcl-2;
- (b) carboxy-terminal truncated versions of (i) naturally occurring Bcl-x<sub>L</sub> or Bcl-2 or (ii) fusion proteins containing Bcl-x<sub>L</sub> or Bcl-2;
- (c) polypeptides containing the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4; or
- (d) peptides that inhibit binding of cytochrome C to a member of the Bcl-2-related family.

10       The truncated polypeptides described in (b) supra typically lack 21 carboxy-terminal amino acids. It is understood that they may lack no more than fifty, preferably forty and more preferably thirty (e.g. twenty seven or twenty three) carboxy-terminal amino acids. Methods to test  
15 for the utility of these truncated polypeptides in the screening assays of the invention are described infra.

In the description of the assays infra, "Polypeptide B" means any one of these molecules. The test compound may be a defined, isolated and purified candidate compound  
20 (e.g., a synthetic small molecule) or may be present in a biological sample such as a biological fluid, tissue extract, subcellular fraction or cellular lysate.

In one aspect of the invention, cytochrome C is provided bound to a solid support. Examples of solid  
25 supports are given infra. Unbound cytochrome C is removed from the solid support (e.g., by washing) and the bound cytochrome C is contacted with detectably labeled "Polypeptide B" in the presence of the test compound. Methods for detectably labeling polypeptides are described  
30 infra. After co-incubation, unbound substances are removed and any "Polypeptide B" that remains bound is detected by any number of techniques well-known in the art, examples of which are described infra.

- 19 -

Instead of using detectably labeled "Polypeptide B", an unlabeled "Polypeptide B" can be used. Bound "Polypeptide B" can then be detected by addition of a detectably labeled antibody that binds specifically to "Polypeptide B". After 5 incubation, unbound antibody is removed and any antibody that remains bound is detected by any one of the methods described infra. The detectably labeled antibodies include those that react specifically with Bcl-x<sub>L</sub>, Bcl-2, any component of a fusion protein containing Bcl-x<sub>L</sub> or Bcl-2, or 10 an epitope within the amino acid sequence of SEQ ID NO:3 or SEQ ID:NO:4.

Instead of using a detectably labeled antibody that specifically binds to "Polypeptide B", an unlabeled antibody can be used. After incubation, unbound antibody is removed 15 and a detectably labeled "secondary" antibody that specifically recognizes the first antibody is added. After incubation and removal of unbound detectably labeled secondary antibody, the presence of the bound detectably labeled secondary antibody is detected by one of the methods 20 described infra.

Alternatively, "Polypeptide B" may be conjugated to biotin by methods known to those of average skill in the art. In this case, bound biotinylated "Polypeptide B" is contacted with detectably labeled avidin and avidin which 25 remains bound to the biotin and thus to the solid support after washing is detected by one of the methods described infra.

Instead of using biotinylated "Polypeptide B", antibody that binds specifically to "Polypeptide B" may be 30 conjugated to biotin. In this case, bound biotinylated antibody is contacted with detectably labeled avidin and avidin which remains bound to the biotin and thus to the

- 20 -

solid support is detected by one of the methods described infra.

In this assay, inhibition of binding of "Polypeptide B" to cytochrome C by a test compound is an indication that 5 the test compound will permit release of cytochrome C from the mitochondria into the cytosol, and thus act as a promoter of apoptosis. On the other hand, enhancement of binding of "Polypeptide B" to cytochrome C by a test compound is an indication that the test compound is an 10 inhibitor of apoptosis.

In another aspect of the invention, "Polypeptide B" is provided fixed to the solid support. This "Polypeptide B" is contacted with soluble cytochrome C in the presence of a test compound. Cytochrome C bound to "Polypeptide B" is 15 detected, for example, by either: (a) the cytochrome C being detectably labeled; (b) unlabeled cytochrome C and a detectably labeled anti-cytochrome C antibody; (c) unlabeled cytochrome C, unlabeled anti-cytochrome C antibody and a detectably labeled "secondary" antibody that binds 20 specifically to the anti-cytochrome C antibody; (d) the cytochrome C being biotinylated and detectably labeled avidin; or (e) unlabeled, unconjugated cytochrome C, biotinylated anti-cytochrome C antibody and detectably labeled avidin, including the same steps for removal of 25 unbound material described infra.

In this assay, inhibition of binding of cytochrome C to "Polypeptide B" by a test compound is again an indication that the test compound is a promoter of apoptosis.

Conversely, enhancement of binding of "Polypeptide B" by a 30 test compound is an indication that the test compound is an inhibitor of apoptosis.

In these assays, compounds that inhibit binding of "Polypeptide B" and cytochrome C could act by direct

- 21 -

competition for binding sites on either polypeptide molecule. Alternatively, they could act by binding at a site on either polypeptide remote from the binding site and affect binding by inducing conformational changes in the 5 relevant polypeptide. Compounds that enhance binding between the two polypeptides are proposed to act by the latter mechanism. However, the present invention is not limited by a particular mechanism of action.

These assays may be performed in the absence or 10 presence of sources of potentially relevant co-factors. Sources include but are not restricted to serum, plasma, tissue extracts, cell lysates, cytosolic extracts or mitochondrial extracts. Those skilled in the art will be able to determine operative and optimal assay conditions for 15 each determination by employing routine experimentation.

Methods to test for the utility of truncated polypeptides lacking more than twenty one carboxy-terminal amino acids (see supra on page 18) in the screening assays of the invention include, but are not restricted to, 20 performing the screening assays in the absence of the test compound. "Polypeptide B" in such assays would be:  
(a) a test truncated polypeptide; or  
(b) the equivalent polypeptide lacking twenty one carboxy-terminal amino acids.

25 In these assays, if the binding of the test truncated polypeptide is as efficient, or more efficient, as the binding obtained with the polypeptide lacking twenty one carboxy-terminal amino acids, the test truncated polypeptide may be used in the screening assays of the invention.

30 Test compounds showing either inhibitory or enhancing activity in these screening assays can be further tested for their ability to regulate cytosolic cytochrome C levels, cleavage of PARP into apoptotic fragments and

- 22 -

apoptosis in cells upon exposure to an appropriate stimulus, e.g. exposure to U-937 cells overexpressing Bcl-X<sub>L</sub> to IR.

Well known solid supports that may be used for screening assays of the invention include, but are not 5 restricted to, glass, polystyrene, polypropylene, polyethylene, dextran, nylon, natural and modified celluloses, and polyacrylamides. In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component can be immobilized by non-covalent or 10 covalent attachments. Non-covalent attachment can be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized can be used to 15 anchor the protein to the solid surface. The surfaces can be prepared in advance and stored.

One of the ways in which a protein (e.g., "Polypeptide B", cytochrome C, an antibody or some other "secondary" reagent such as avidin) can be detectably 20 labeled is by linking it to an enzyme for use in an enzyme immunoassay (EIA) [Voller (1978), The Enzyme Linked Immunosorbent Assay (ELISA), Diagnostic Horizons 2:1 (Microbiological Associates Quarterly Publication, Walkersville, MD); Voller, et al. (1978) J. Clin. Pathol. 25 31:507; Butler (1981) Meth. Enzymol. 73:482; Maggio (ed.) (1980) Enzyme Immunoassay, CRC Press, Boca Raton, FL; Ishikawa, et al. (eds.) (1981) Enzyme Immunoassay, Kgaku Shoin, Tokyo]. The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a 30 chromogenic substrate, in such a manner as to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorimetric or visual means. Enzymes that can be used to detectably label the antibody include,

- 23 -

but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods that employ a chromogenic substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection can also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling appropriate polypeptides, it is possible to detect bound material through the use of a radioimmunoassay (RIA) [see, for example, Weintraub (1986), Principles of Radioimmunoassay, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society]. The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter, or by autoradiography.

It is also possible to label with a fluorescent compound. When the fluorescently labeled material is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin,  $\alpha$ -phthaldehyde and fluorescamine.

Proteins can also be detectably labeled using fluorescence emitting metals such as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as

- 24 -

diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The polypeptides also can be detectably labeled by coupling to a chemiluminescent compound. The presence of 5 the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminal, isoluminol, theromatic acridinium ester, imidazole, 10 acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the relevant proteins. Bioluminescence is a type of chemiluminescence found in biological systems, in which a catalytic protein increases the efficiency of the 15 chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase, and aequorin.

20 C.1 Sources of peptides and polypeptides

Peptides and polypeptides used in the screening assays of the invention may be obtained by a variety of means. Smaller peptides (less than 50 amino acids long) may be conveniently synthesized by standard chemical methods. 25 Some polypeptides (e.g. cytochrome C antibodies) may be purchased from commercial sources. Modified forms of cytochrome C, which may be used in the assays of the invention (e.g., apocytochrome C and porphyrin cytochrome C), can be either purchased from commercial sources or 30 chemically derived by methods familiar to those of average skill in the art. Where otherwise unavailable, antibodies can be generated as described in Section D infra. Detectably labeled antibodies either can be purchased from

- 25 -

commercial sources or are readily prepared by those of ordinary skill in the art.

Polypeptides such as Bcl-x<sub>L</sub> and Bcl-2 may be purified from biological sources by methods well-known to 5 those skilled in the art (Protein Purification, Principles and Practice, second edition (1987) Scopes, Springer Verlag, N.Y.). They may also be produced in their naturally occurring, truncated, or fusion protein forms by recombinant DNA technology using techniques well known in the art. These 10 methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.,; and Ausubel et al., 15 eds. (1989), Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., N.Y.. Alternatively, RNA encoding the proteins may be chemically synthesized. See, for example, the techniques described in Oligonucleotide Synthesis, (1984) Gait, M.J. 20 ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the nucleotide sequences. Where the peptide or polypeptide is soluble, it can be recovered from: 25 (a) the culture, i.e., from the host cell in cases where the peptide or polypeptide is not secreted; or (b) from the culture medium in cases where the peptide or polypeptide is secreted by the cells. The expression systems also encompass engineered host cells that express the polypeptide 30 *in situ*, i.e., anchored in the cell membrane. Purification or enrichment of the polypeptide from such an expression system can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in

- 26 -

the art. Alternatively, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the protein, but also to assess  
5 biological activity.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (for example, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA,  
10 plasmid DNA or cosmid DNA expression vectors containing the nucleotide sequences; yeast transformed with recombinant yeast expression vectors; insect cells infected with recombinant viral expression vectors (baculovirus); plant cell systems infected with recombinant viral expression  
15 vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors; or mammalian cells (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian  
20 cells (e.g. metallothionein promoter) or from mammalian viruses.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the gene product being expressed. For example,  
25 when a large quantity of such a protein is to be produced, e.g. for raising antibodies to the protein, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli*  
30 expression vector pUR278 [Ruther et al. (1983) EMBO J. 2:1791], in which the coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors

- 27 -

[Inouye & Inouye (1985) Nucleic Acids Res. 13:3101; Van Heeke & Schuster (1989) J. Biol. Chem. 264:5503]; and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione

5 S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage  
10 sites so that the cloned target gene product can be released from the GST moiety.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the nucleotide  
15 sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region  
20 of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the gene product in infected hosts [e.g., See Logan & Shenk (1984) Proc. Natl. Acad. Sci. USA 81:3655]. Specific initiation signals may also be required for efficient  
25 translation of inserted nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional  
30 translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be

provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be 5 of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. [Bittner et al. (1987) Methods in Enzymol. 153:516].

10 In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be 15 important for the function of the protein. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. Mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

20 For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the sequences described above may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be 25 transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 30 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and

- 29 -

grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the gene product. Such engineered cell lines may be particularly useful in

- 5 screening and evaluation of compounds that affect the endogenous activity of the gene product.

A fusion protein may be readily purified by utilizing an antibody or a ligand that specifically binds to the fusion protein being expressed. For example, a system  
10 described by Janknecht et al. [(1991) Proc. Natl. Acad. Sci. USA 88:8972] allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a  
15 vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni<sup>2+</sup> nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-  
20 containing buffers. If desired, the histidine tag can be selectively cleaved with an appropriate enzyme.

**D. ANTIBODIES TO EPITOPES WITHIN THE AMINO ACID SEQUENCES OF SEQ. ID NOS:1, 2, 3 AND 4.**

Antibodies that specifically recognize epitopes  
25 within the amino acid sequence of SEQ. ID NOS:1, 2, 3 and 4 are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub>,  
30 fragments, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of the Bcl-x<sub>L</sub> or Bcl-2 in a

- 30 -

biological sample, and may therefore be utilized as part of a diagnostic or prognostic technique whereby patients are tested for abnormal amounts of Bcl-x<sub>L</sub> or Bcl-2. Such antibodies may also be utilized in the screening assays of  
5 the invention.

For the production of antibodies of the invention, a host animal is immunized by injection with a peptide containing the amino acid sequence of SEQ. ID NOS:1, 2, 3 and 4. Such host animals may include but are not limited to  
10 rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete) adjuvant, mineral gels such as aluminum hydroxide, lysolecithin,  
15 pluronic polyols, polyanions, peptides, oil emulsions, BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.  
20

In order to further enhance immunogenicity, the immunogen may be coupled to a carrier. Examples of such carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used  
25 as carriers. Methods of coupling a peptide to a carrier are well known in the art and include the use of glutaraldehyde, carbodiimide and m-maleimidobenzoyl-N-hydroxysuccinimide ester.

Since epitopes within SEQ ID NOS:1, 2 and 3 and SEQ  
30 ID NO:4 are predicted to interact with cytochrome C, they would be exposed on the surface of Bcl-x<sub>L</sub> and Bcl-2, respectively. Therefore, they would not be predicted to be "hidden" epitopes when immunizing with intact Bcl-x<sub>L</sub> and

- 31 -

Bcl-2. Furthermore, since Bcl-x<sub>L</sub> and Bcl-2 are intracellular proteins and thus normally sequestered from the immune system, neither CD4<sup>+</sup> helper T-cells nor B-cells of the host animal will likely be tolerant of the epitopes 5 within SEQ ID NOS:1, 2, 3 and 4. Therefore, the host animal is unlikely to be immunologically tolerant and thus unresponsive to the immunogen. The amount of antigen to be used can be determined readily by those with average skill in the art without undue experimentation. The antigen can 10 be administered by a number of routes (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various time points after administration. When the desired 15 level of antibody is obtained, the animal is bled and the serum is stored.

Monoclonal antibodies (mAb), which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production 20 of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique [Kohler and Milstein (1975) *Nature* 256:495-497; U.S. Patent No. 4,376,110; Howell and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Press, 25 N.Y.], the human B-cell hybridoma technique [Kosbor et al. (1983) *Immunology Today* 4:72; Cole et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:2026], and the EBV-hybridoma technique [Cole et al. (1985), Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc.]. Such antibodies may be of any 30 immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

In addition, techniques developed for the production of "chimeric antibodies" can be used [Morrison et al. (1984)

- 32 -

Proc. Natl. Acad. Sci. USA 81:6851; Neuberger et al. (1984) Nature 312:604; Takeda et al. (1985) Nature 314:452]. These involve splicing a portion of a gene encoding a mouse antibody of appropriate antigen specificity to a portion of 5 a gene encoding a human antibody of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

10 Alternatively, techniques described for the production of single chain antibodies [U.S. Patent 4,946,778; Bird (1988) Science 242:423; Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879; and Ward et al. (1989) Nature 334:544] can be adapted to produce single chain 15 antibodies against the epitopes of SEQ ID NOS:1, 2, 3 and 4. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. They are conveniently produced by recombinant DNA techniques.

20 Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to the F(ab')<sub>2</sub>, fragments which can be produced by pepsin digestion of the antibody molecule, and the Fab fragments which can be 25 generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed [Huse et al. (1989) Science 246:1275] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

30 Methods for screening antibodies for binding specificity are well known. These include but are not restricted to testing for: (a) binding to a peptide containing the amino acid sequence of SEQ ID NOS:1, 2, 3 and

- 33 -

4; (b) lack of binding to peptides and polypeptides lacking these sequences; and (c) inhibition of binding to intact Bcl-x<sub>L</sub> and Bcl-2 by peptides containing the amino acid sequence of SEQ ID NOS:1, 2, 3 and 4.

5

#### E. THERAPY

The invention includes methods for producing a medically desirable effect in a patient by either enhancing apoptosis or inhibiting apoptosis. Patients that would benefit from promotion of apoptosis include but are not limited to those with, or at least suspected of having:

(a) autoimmune diseases in which it is desirable to enhance the death of the specifically activated, apoptosis-susceptible lymphocytes (T and/or B) which are the effectors of the tissue destruction resulting in the symptoms of the disease;

(b) allo- or xenograft transplant recipients in which an optimal therapeutic modality results in the elimination of the effector cells, including activated, apoptosis-susceptible, graft-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes, whose activity results in rejection of the graft; and

(c) cancer patients in which therapy is aimed at cytolysis of the tumor cells.

Patients that would benefit from inhibition of apoptosis include but are not limited to those with, or at least suspected of having:

(d) autoimmune diseases in which affected tissues are protected from the destructive activities of the effector cells of the immune system, these activities substantially involving apoptosis of affected tissue cells;

(e) xeno- and allograft transplant patients in which, similar to the situation described in (d) supra, it is desirable to protect the cells of the grafted tissue from

- 34 -

apoptosis-inducing action of the immunological effector cells; and

(f) patients with immunodeficiency diseases such as AIDS in which a large proportion of the disease-related CD4<sup>+</sup> T-cell depletion involves apoptosis.

Therapeutic methods of the invention that result in enhancement of apoptosis involve the use of peptides of the invention. These include all those described in Section B supra.

10 Therapeutic methods of the invention that result in inhibition of apoptosis utilize a polypeptide (or a nucleic acid that encodes a polypeptide) that includes multiple peptide domains, each of which contains the amino acid sequence of SEQ ID NO:3.

15 E.1 Methods of enhancing apoptosis

In methods of the invention that enhance apoptosis, a peptide or peptidomimetic that inhibits binding of cytochrome C to an anti-apoptotic Bcl-2-related family member (such as a polypeptide containing the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4) is delivered to relevant cells. Delivery involves administering to a patient either the peptide or peptidomimetic itself, a nucleic acid encoding the peptide, an expression vector encoding the peptide, or cells transfected or transduced 25 with the vector.

*E.1.1 Administration of a peptide*

Peptides and peptidomimetics may be delivered directly to cells *in vitro*. Methods of achieving this are well known to those of average skill in the art.

30 Furthermore, peptides may be delivered to a cell of a mammal using techniques substantially the same as those described infra for delivery to human patients. Examples of appropriate mammals include but are not restricted to

- 35 -

humans, non-human primates, horses, cattle, sheep, dogs, cats, mice, rats, guinea pigs, hamsters, rabbits and goats.

A peptide of the invention may be delivered to cells of a patient in its unmodified state, dissolved in an appropriate physiological solution, e.g. physiological saline. Alternatively, it may be modified as detailed in Section B.2 in order to facilitate transport across cell and/or intracellular membranes and to prevent extracellular or intracellular degradation. Its transport across biological membranes may also be enhanced by delivering it encapsulated in liposomes [Gabizon et al. (1990) *Cancer Res.* 50:6371; Ranade (1989) *J. Clin. Pharmacol.* 29:685] or an appropriate biodegradable polymeric microparticle (also referred to as a "microsphere", "nanosphere", "nanoparticle, or "microcapsule"). Naturally, it is desirable that these peptides be selectively targeted to relevant tissues and cell types. This can be achieved by contacting the peptides directly with the affected organ or tissue, e.g., by localized injection or implantation. Thus, in autoimmune diseases such as rheumatoid arthritis (RA) or insulin-dependent diabetes mellitus (IDDM), the peptides could be introduced directly into affected joints or the pancreas, respectively, or, preferably, into draining lymphoid tissue in which the active autoimmune response occurs. The latter procedure would obviate the potential tissue damage caused by introducing pro-apoptotic peptides into the cells of the target organ.

Alternatively, peptides of the invention may be delivered in liposomes into which have been incorporated ligands for receptors on relevant cells (e.g., T cells or B cells) or antibodies to cell-surface markers expressed by these cells. Thus an antibody specific for the CD4 T cell surface marker may direct liposomes containing both the

- 36 -

anti-CD4 antibody and the relevant pro-apoptotic peptide to a CD4<sup>+</sup> T cell. In autoimmune diseases in which the T cell receptor (TCR) expressed by a dominant pathogenic T-cell clone has been defined, an antibody specific for the 5 relevant TCR component (e.g. V $\beta$ ) may be used. The latter methodology would represent an ideal form of immunotherapy in which pathogenic effector cells are specifically targeted for elimination while the immune system as a whole and the cells of the target organ remain uncompromised. The same 10 approach of targeting T-cells, both CD4<sup>+</sup> and CD8<sup>+</sup>, could be used in transplant recipients.

In cancer patients, the pro-apoptotic peptides are preferably directed to cancer cells. The peptides could, for example, be injected directly into the tissues 15 surrounding the tumor site after surgery to remove the tumor, in order to eliminate residual tumor cells. Instead of surgery, the tumor could be treated by *in situ* injection of the peptide into the tumor. The treatment can be combined with radiotherapy, chemotherapy or immunotherapy to 20 enhance tumor cell apoptosis. The liposome methodology described supra, could also be exploited. In this case antibodies specific for tumor-specific antigens (TSA) or tumor-associated antigens (TAA) would be exploited.

It is well known in the medical arts that dosages 25 for any one patient depend on many factors, as well as the particular compound to be administered, the time and route of administration and other drugs being administered concurrently. Dosages for the peptides of the invention will vary, but can be, when administered intravenously, 30 approximately 0.01 mg to 10 mg/ml blood volume. Routes and doses of administration are well known to skilled pharmacologists and physicians. Routes, in addition to those described supra, include, but are not restricted to:

- 37 -

intraperitoneal, intramuscular, intrapulmonary,  
transmucosal, subcutaneous and intravenous.

*E.1.2 Administration of peptides utilizing expression  
vectors encoding the peptides.*

5 An expression vector is composed of or contains a nucleic acid in which a polynucleotide sequence encoding a peptide or polypeptide of the invention is operatively linked to a promoter or enhancer-promoter combination. A promoter is a transcriptional regulatory element composed of  
10 a region of a DNA molecule typically within 100 nucleotide pairs in front (upstream of) of the point at which transcription starts. Another transcriptional regulatory element is an enhancer. An enhancer provides specificity in terms of time, location and expression level. Unlike a  
15 promoter, an enhancer can function when located at variable distances from the transcription site, provided a promoter is present. An enhancer can also be located downstream of the transcription initiation site. A coding sequence of an expression vector is operatively linked to a transcription  
20 terminating region. To bring a coding sequence under control of a promoter, it is necessary to position the translation initiation site of the translational reading frame of the peptide or polypeptide between one and about fifty nucleotides downstream (3') of the promoter. Examples of  
25 particular promoters are provided infra. Expression vectors and methods for their construction are known to those familiar with the art.

Suitable vectors include plasmids, and viral vectors such as herpes viruses, retroviruses, canary pox viruses,  
30 adenoviruses and adeno-associated viruses, among others.

The application of peptide-encoding genes to the treatment of cancer, autoimmune disease, or graft rejection

in humans can utilize either *in vivo* or *ex vivo* based therapeutic approaches.

The *in vivo* approach requires delivery of a genetic construct directly into the patient, preferably targeting it 5 to the cells or tissue of interest. For example, after surgical removal of a primary tumor, residual cells may be targeted by treating the vicinity of the tumor with a composition containing a retroviral vector encoding an immunostimulatory peptide. Alternatively, instead of 10 surgery, the primary tumor could be treated by *in situ* injection of the vector directly into the tumor. Malignant cells distal to the primary tumor site may be reached by delivering the vector intravenously. Similarly, targeting of tissues under autoimmune attack may be achieved by direct 15 injection of vectors. Targeting of tumor cells or activated lymphocytes, for example, can be accomplished by the use of a retrovirus, which stably transfets primarily proliferating cells.

Tissue specific targeting may also be achieved by 20 the use of a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on tumor cells [Cristiano et al. (1995) J. Mol. Med 73:479]. Similarly, tumor and cell specific 25 antibodies of the type described supra in Section E.1.1 can be bound to vectors and thereby target them to tumors or cells such as T-lymphocytes. The latter would be useful in both autoimmune diseases and organ transplantation. A promoter inducing relatively tumor-specific expression can 30 be used to achieve a further level of targeting: for example, the  $\alpha$ -fetoprotein promoter for hepatocellular carcinoma [Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039]; the DF3 tumor antigen promoter/enhancer for

- 39 -

certain breast and lung carcinomas [Abe and Kufe (1993) Proc. Natl. Acad. Sci. USA 90:282] or the tyrosinase promoter for melanoma [Hart et al. (1995) Br. Med. Bull. 51:647]. Tissue-specific promoters for use in autoimmune or 5 transplant patients include, for example, the inducible IL-2 [Thompson et al. (1992) Mol. Cell. Biol. 12: 1043], IL-4 [Todd et al. (1993) J. Exp. Med. 177:1663] and gamma-interferon [Penix et al. (1993) J. Exp. Med. 178:483] T-cell targeting promoters. Such inducible promoters would have an 10 invaluable additional advantage in that expression would occur selectively in activated T-cells. Included in this population of activated T-cells are the effector cells that an ideal immuno-therapeutic modality would selectively eliminate in autoimmune and transplant patients.

15 Vectors can also be delivered by incorporation into liposomes or other delivery vehicles either alone or co-incorporated with cell specific antibodies, as described supra in Section E.1.1 for pro-apoptotic peptides.

All of the above cancer therapeutic methods may be 20 used in conjunction with chemotherapeutic agents such as CDDP (see Example 1 infra), radiation therapy (see Example 1 infra), or immunotherapy, all of which act substantially by inducing apoptosis in the tumor cells. Such therapy combinations may result in synergistic or at least additive 25 therapeutic effects.

DNA or transfected cells may be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to a human, e.g., 30 physiological saline. A therapeutically effective amount is an amount of the DNA of the invention which is capable of producing a medically desirable result in a treated animal. As is well known in the medical arts, the dosage for any one

- 40 -

patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently.

- 5 Dosages will vary, but a preferred dosage for intravenous administration of DNA is from approximately  $10^6$  to  $10^{12}$  copies of the DNA molecule. This dose can be repeatedly administered, as needed. Routes of administration will be as for the peptides described supra in Section E.1.1.
- 10 E.2 Methods of inhibiting apoptosis

In methods of the invention that diminish apoptosis, a recombinant polypeptide containing multiple peptide domains, each of which contains the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4, is introduced into the cells and 15 preferably into the mitochondria of cells of a relevant tissue. The polypeptide includes at least two copies of the domain, and preferably at least three (e.g., four, five, six, eight, ten, twelve, or twenty). Each domain can be represented by SEQ ID NO:3 or 4, or can be a larger segment 20 or all of  $\Delta X_{L/S}$  or the corresponding portion of Bcl-2. These recombinant polypeptides, in which the above described domains are either present in tandem on the polypeptide molecule or are separated by stretches of other polypeptide sequence (e.g. other Bclx or Bcl-2 sequence), would display 25 an enhanced ability to bind cytochrome C, prevent its entry into the cytosol, and thereby render the cells relatively more resistant to apoptosis induction.

The recombinant polypeptide is preferably introduced into the mitochondria of the patient by administering to the 30 patient a nucleic acid of the invention that encodes the polypeptide. Targeting to the mitochondria would be accomplished by: (a) employing an appropriate signal sequence in construction of the nucleic acid encoding the

- 41 -

polypeptide (e.g., utilizing the same mechanism utilized by the cell to deliver other proteins such as Bcl-x<sub>L</sub> into the mitochondria); and (b) inserting nucleic segments encoding the amino acid sequences of SEQ ID NO:3 or SEQ ID NO:4 into 5 a nucleic acid encoding Bcl-x<sub>L</sub> or Bcl-2 at positions predicted from the known structure of Bcl-x<sub>L</sub> [Muchmore et al., cited supra] and Bcl-2 [Vance et al., cited supra] to encode regions on the outer surface of the polypeptide and thereby be available for interaction with cytochrome C.

10 An example of an appropriate signal sequence as described in (a) would be a naturally-occurring Bcl-x<sub>L</sub> or Bcl-2 signal sequence. Vehicles of delivery (vectors), methods of administration and methods of tissue-specific targeting are substantially the same as those used for *in* 15 *vivo* administration of expression vectors encoding peptides that promote apoptosis (see Section E.1.2 supra).

This method of the invention may be used to inhibit apoptosis in tissues undergoing autoimmune attack or transplanted organs undergoing rejection. Naturally, it 20 would again be necessary to target the nucleic acid to cells of the organ experiencing autoimmune destruction or immunologic rejection and to avoid infecting, transfected, or transducing immune cells mediating the deleterious immune response. Targeting the latter would inhibit their 25 apoptosis and thereby enhance their tissue destructive potential. By way of example, it would be possible to target the nucleic acid encoding the anti-apoptotic polypeptide to synovial cells in RA patients by conjugating it with an antibody that binds to a cell-surface molecule on 30 such cells, and thereby protect the synovial cells from the lethal effects of the effector lymphocytes and inflammatory cytokines involved in the pathogenesis of RA. A vector comprising an insulin promoter [Dandoy et al. (1991) Nucleic

- 42 -

Acids Res. 19:4925; Selden et al. (1986) Nature 321:525] would target expression of the apoptosis inhibiting polypeptide to pancreatic islet cells and would thereby selectively protect them from the pathologic immunologic and 5 inflammatory activities involved in autoimmune IDDM. Cardiac myosin [Lee et al. (1992) J. Biol. Chem. 267:15875], renin [Fukamizu et al. (1994) Biochem. Biophys. Res. Commun. 179:183] and transferrin [Idzerda et al. (1989) Mol. Cell. Biol. 9:5154] promoters could similarly be used to protect 10 grafted hearts, kidneys and livers, respectively, from immunological rejection.

CD4<sup>+</sup> T-cell specific antibodies and promoters, examples of which were given in Sections E.1.1 and E.1.2. supra, could direct expression of the anti-apoptotic 15 polypeptide to these T-cells in certain immunodeficiency patients, for example, patients that are either HIV-1 positive or have AIDS. This therapeutic method would substantially preserve these CD4<sup>+</sup> T-cells which are absolutely essential for a functional immune system and the 20 loss of which leads to the devastating immunodeficiency observed in AIDS patients. Targeting of the nucleic acid encoding the anti-apoptotic polypeptide to bone marrow with a pluripotent stem cell-specific antibody (e.g., anti-CD34 antibody) would also substantially preserve the 25 hematological systems of cancer patients undergoing chemo- and/or radiation therapy. Bone marrow depletion and consequent hematological insufficiency is a frequent side-effect of these therapies. Directing expression of the anti-apoptotic polypeptide to cells of the neurological system 30 would be useful in treatment of certain neurodegenerative conditions resulting from excessive neurological cell death.

- 43 -

The following examples are meant to illustrate the invention and not to limit it.

#### EXAMPLES

##### Materials and Methods

###### 5 Cell culture and reagents

Human U-937, U-937/Bcl-x<sub>L</sub> and U-937/p35 [Datta et al. (1997), cited supra] myeloid leukemia cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml 10 streptomycin and 2 mM L-glutamine. Irradiation was performed with a γ-ray source (Cesium 173, Gamma Cell 1000, Atomic Energy of Canada, Ltd., Ontario) at a fixed dose rate of 13 Gy/min. Cells were also treated with 50 µM cisplatin (CDDP) (Sigma, St. Louis, MO) or with 1 mM methyl methanesulfonate 15 (MMS) (Sigma).

###### Isolation of the cytosolic fraction

Cells were washed twice with PBS and the pellet was suspended in 5 ml of ice cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 0.1mM PMSF, 10 µg/ml leupeptin, aprotinin, and pepstatin A) containing 250 mM sucrose. The cells were homogenized by douncing 3 times in a Dounce homogenizer with a sandpaper polished pestle. After centrifugation for 5 min at 40°C, the supernatants were then centrifuged at 105,000g for 30 min at 25 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

###### Immunoprecipitation and immunoblot analysis

Cell lysates were prepared as described [Kharbanda et al. (1994) J. Biol. Chem. 269:20739]. 30 Immunoprecipitations were performed using anti-Bcl-x (Novartis, NJ) or anti-cytochrome C antibodies [Kirken et al. (1995) Protein Expression and Purification 6:707]. Proteins were separated by sodium dodecyl sulfate

- 44 -

polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and probed with the indicated antibodies. The blots were developed by enhanced chemiluminescence (ECL) (Amersham). Preparation of lysates and immunoblotting for 5 PARP were carried out as described, using the c-2-10 anti-PARP monoclonal antibody [Desnoyers et al. (1994) Annals Biochem. 218:470].

Immunoelectron microscopy on frozen cell sections

U-937 cells were resuspended in Tyrode buffer, 10 pH 7.4, and fixed in 8% paraformaldehyde in phosphate buffer, as described [Israels et al. (1992) Blood 80:143]. The cells were then incubated with 20% poly(vinyl pyrrolidone) in 1.84 M sucrose, mounted on aluminum pins and stored in liquid nitrogen until sectioned using the method 15 of Stenberg et al. [(1984) Histochem J. 16:983]. Frozen thin sections were incubated with primary antibodies (anti-Bcl-x or anti-cytochrome C) followed by protein-A or goat anti-rabbit IgG conjugated to 14 nm gold particles. Parallel control samples included substitution of buffer or non- 20 immune serum for the primary antibody. Following labeling, the sections were stained with poly(vinyl alcohol) and uranyl acetate. Sections were examined using transmission electron microscopy.

Generation of Bcl-x<sub>LT</sub> and Bcl-x<sub>ST</sub> proteins. Full length Bcl-x<sub>L</sub> 25 and Bcl-x<sub>S</sub> were cloned from a cDNA library of human Raji lymphoma cells (Clontech) using the PCR primers: 5'-primer: 5'-GGAATTCAATATGTCTCAGAGCAACCG-3' (SEQ ID NO:15); 3'-primer: 5'-AGAATTCAATTCCGACTGAAGAG-3' (SEQ ID NO:16). Following cloning into the TA vector (Invitrogen), inserts were 30 subcloned into the EcoRI site of pProEX (Gibco/BRL) to generate His-tagged fusion constructs or pGEX-3T to generate GST fusion proteins. Truncated Bcl-x proteins (Bcl-x<sub>LT</sub> or Bcl-x<sub>ST</sub>) lacking 21 amino acids from the C-terminus were

- 45 -

generated by PCR cloning using the 5'-primer described above and the 3'-primer: 5'-ATCGAATTCATCAGCGGTTGAAGCGTTCC-3' (SEQ ID NO:17). All other cloning steps to generate truncated proteins were performed as described for full-length 5 proteins. GST-Bcl-x<sub>ST</sub> was cleaved with thrombin to generate Bcl-x<sub>ST</sub>. Folding and protein solubility were verified by circular dichroism and dynamic light scattering measurements. Fusion proteins were expressed as soluble proteins in E. coli and purified as described [Polayes and 10 Hughes (1994) Focus 16:81].

Far-Western analysis

Column-purified H<sub>6</sub>-Bcl-x<sub>LT</sub>, Bcl-x<sub>ST</sub> and H<sub>6</sub> polypeptides were resolved by SDS-PAGE and transferred to a nitrocellulose filter. Purified MAP kinase (MAPK) protein 15 was used as a negative control. The filter was incubated with purified cytochrome C (bovine heart cytochrome C; Sigma) for 1 h at room temperature. The filters were then analyzed by immunoblotting with anti-cytochrome C antibody.

In separate experiments, purified cytochrome C was 20 separated by SDS-PAGE and transferred to three nitrocellulose filters. The filters were incubated with purified, eluted H<sub>6</sub>, H<sub>6</sub>-Bcl-x<sub>LT</sub> or Bcl-x<sub>ST</sub> for 1 h at room temperature. The filters were then analyzed by immunoblotting with anti-Bcl-x antibody.

25 ELISA immunoassay

Cytochrome C was adsorbed onto the plastic surface of a 96-well ELISA plate, followed by addition of blocking buffer (Superblock™, Pierce Chemical) to reduce non-specific binding. Glutathione-S-transferase (GST), GST-Bcl-x<sub>LT</sub> or 30 GST-Bcl-x<sub>ST</sub> in Tris-buffered saline containing 5% bovine serum albumin and 0.5% NP-40 were added to the wells. After incubation for 2 h at room temperature, the plates were washed and incubated with rabbit anti-GST, followed by

- 46 -

washing and incubation with alkaline phosphatase-conjugated anti-rabbit IgG antibody. The plates were developed using a fluorogenic substrate and reactivity determined by measurement of fluorescence.

5 Peptide synthesis

Peptides were synthesized on an Advanced Chemtech 396 MPS peptide synthesizer (Louisville, KY) using a 9-fluorenylmethyloxycarbonyl (Fmoc) protocol provided by the manufacturer. 2-(1H-benzotriazole-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBr) was used for amino acid couplings and Fmoc cleavages were accomplished with 25% piperidine in dimethylformamide (DMF). Fmoc-Aa-Wang resins (where Aa is the C-terminal amino acid of each sequence, 0.05 mmol, Midwest Biotech, IN) were employed for synthesis of each peptide. Side chains of functional amino acids were protected as Cys(Trt), Asn(Trt), Trp(Boc), Asp(t-Bu), Gln(Trt), Arg(Pbf), Glu(t-Bu), His(Trt), Lys(Boc), Ser(t-Bu), Thr(t-Bu), and Tyr(t-Bu) where tBu is a tert-butyl, Trt is a trityl, Boc is a tert-butyloxycarbonyl, and Pbf is a N<sup>G</sup>-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group. Double couplings were performed for each amino acid (5-fold excess). Completed peptides were cleaved from the resins using trifluoroacetic acid (TFA)/H<sub>2</sub>O/1,2-ethanediol (92.5/5/2.5%). Peptide solutions were concentrated and precipitated with anhydrous diethyl ether, filtered and dried. The peptides were analyzed by RP-HPLC (C<sub>18</sub>, 5% - 95% acetonitrile/0.1% TFA, 30 min. gradient) and characterized by mass spectrometry.

30 Peptide inhibition assay

This assay was a modification of the ELISA immunoassay described supra. All steps were the same except that the GST-Bcl-x<sub>LT</sub> was mixed with synthetic peptide in a

- 47 -

separate plate prior to addition to the assay plate containing the adsorbed cytochrome C. The final concentration of peptide was in the range 1 - 100  $\mu$ M

Example 1

- 5 **Bcl-x<sub>L</sub> blocks cytochrome C release from mitochondria of U-937 cells and cytochrome C release occurs upstream to activation of cysteine proteases**

U-937 cells were exposed to 20 Gy IR to determine whether cytosolic cytochrome C levels change with induction 10 of apoptosis. Cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-cytochrome C antibody. Cytochrome C levels were increased in the cytosol at 3 and 6 h after irradiation. Immunoblotting with anti-PARP antibody revealed that these cells also responded 15 to IR with cleavage of PARP into characteristic apoptotic fragments [Kaufmann et al. cited supra; Lazebnik et al. (1994) *Nature* 371:346]. This treatment also caused induction of DNA fragmentation [Datta et al. (1995), cited supra]. Similar results were obtained with other inducers of 20 apoptosis, including CDDP and MMS. Electron microscopic studies performed with anti-cytochrome C antibodies demonstrated immunogold labeling of mitochondria from control cells, while mitochondrial cytochrome C was depleted following exposure to IR. These results support a mechanism 25 in which release of mitochondrial cytochrome C into the cytoplasm occurs in concert with induction of apoptosis.

U-937 cells that stably overexpress Bcl-x<sub>L</sub> (U-937/Bcl-x<sub>L</sub>) are resistant to radiation-induced internucleosomal DNA fragmentation [Datta et al. (1995), 30 cited supra]. Immunoblotting of electrophoretically separated cytosolic proteins from U-937/Bcl-x<sub>L</sub> and control U-937 cells showed that Bcl-x<sub>L</sub> expression also prevented accumulation of cytosolic cytochrome C in response to IR or

- 48 -

CDDP treatment. Moreover, IR-treated U-937/Bcl-x<sub>L</sub> cells failed to exhibit PARP cleavage as indicated by electrophoresis and immunoblotting of the same samples with anti-PARP antibody. The finding that Bcl-x<sub>L</sub> blocks 5 accumulation of cytosolic cytochrome C and cleavage of PARP raised the possibility that cytochrome C functions upstream of the activation of cysteine proteases. To address this issue, U-937 cells that stably overexpress the cysteine protease inhibitor p35 [Bump et al. cited supra; Datta et 10 al. (1996) Blood 88:1936] were irradiated. Overexpression of p35 had no detectable effect on accumulation of cytosolic cytochrome C, but blocked cleavage of PARP and internucleosomal DNA fragmentation. These findings suggest that cytochrome C release from mitochondria in response to 15 apoptotic stimuli is upstream to activation of cysteine proteases.

Example 2

Cytochrome C associates with Bcl-x<sub>L</sub> but not with Bcl-x<sub>S</sub>, and the binding of cytochrome C to Bcl-x<sub>L</sub> is inhibited by Bcl-x<sub>S</sub> 20 Since Bcl-x<sub>L</sub> blocks the increase of cytosolic cytochrome C, co-immunoprecipitation studies were carried out to determine whether these proteins form a specific complex in cells. Lysates from control and irradiated U-937/Bcl-x<sub>L</sub> cells were subjected to immunoprecipitation with 25 anti-cytochrome C antibody. Immunoblotting analysis of the resulting immunoprecipitates with anti-Bcl-x antibody demonstrated reactivity with Bcl-x<sub>L</sub>, but not Bcl-x<sub>S</sub>, that was independent of IR exposure. The demonstration that Bcl-x<sub>L</sub> associates with cytochrome C is in concert with the 30 finding that Bcl-x protein is detectable within mitochondria, as shown by electron microscopic analysis of U-937 cells stained with anti-Bcl-x<sub>L</sub> antibody followed by goat anti-rabbit IgG conjugated to 14 nm gold particles.

- 49 -

To further demonstrate that Bcl-x<sub>L</sub> interacts directly and specifically with cytochrome C, recombinant hexa-His(H<sub>6</sub>)-Bcl-x<sub>LT</sub> was resolved by gel electrophoresis, transferred to a nitrocellulose filter, and renatured in aqueous buffer. After incubation with purified cytochrome C, the filter was washed and probed with anti-cytochrome C antibody. Reactivity with the anti-cytochrome C antibody at the position corresponding to Bcl-x<sub>L</sub> demonstrated a direct interaction between cytochrome C and Bcl-x<sub>L</sub>. By contrast, there was no detectable binding of cytochrome C to Bcl-x<sub>ST</sub> or MAPK. The absence of reactivity with Bcl-x<sub>S</sub> or MAPK indicated that binding of cytochrome C to Bcl-x<sub>L</sub> is specific. Purified cytochrome C was also resolved by electrophoresis and transferred to nitrocellulose filters, which were then incubated with purified H<sub>6</sub>, H<sub>6</sub>-Bcl-x<sub>LT</sub> or Bcl-x<sub>ST</sub> proteins and analyzed by immunoblotting with anti-Bcl-x antibody. Only the blot incubated with H<sub>6</sub>-Bcl-x<sub>LT</sub> showed reactivity with cytochrome C. Similar results were obtained by an ELISA in which purified cytochrome C adsorbed to wells was incubated with GST (▲), GST-Bcl-x<sub>LT</sub>(△) or GST-Bcl-x<sub>ST</sub> (■) (Fig. 1A). After development with a fluorogenic substrate, only wells containing GST-Bcl-x<sub>L</sub> produced a fluorescent product.

Since these results support binding of cytochrome C to Bcl-x<sub>L</sub> but not Bcl-x<sub>S</sub>, an experiment was carried out to determine whether Bcl-x<sub>S</sub>, which can form a complex with Bcl-x<sub>L</sub>, blocks the interaction between cytochrome C and Bcl-x<sub>L</sub>. Cytochrome C adsorbed to plates was incubated with 1 µg/ml GST, 1 µg/ml GST-Bcl-x<sub>LT</sub>, 5 µg/ml GST-Bcl-x<sub>ST</sub>, or a mixture of 1 µg/ml GST-Bcl-x<sub>LT</sub> and 5 µg/ml GST-Bcl-x<sub>ST</sub>. Binding of Bcl-x (Fig. 1B) was assessed by ELISA as in Fig. 1A. Hatched bars represent controls with no cytochrome C. Solid bars represent assays performed in the presence of

- 50 -

cytochrome C. The results demonstrate that addition of excess Bcl-x<sub>s</sub> inhibits binding of cytochrome C to Bcl-x<sub>L</sub>. These findings with recombinant proteins confirm that the binding between cytochrome C and Bcl-x<sub>L</sub> found by co-  
5 immunoprecipitation of cellular lysates is due to a specific interaction which can be blocked by Bcl-x<sub>s</sub>.

Example 3

**Peptides from the  $\Delta X_{L/s}$  region of Bcl-x<sub>L</sub> inhibit binding of cytochrome C to Bcl-x<sub>L</sub>**

10 In light of the observations that 1) cytochrome C binds to Bcl-x<sub>L</sub> but not to Bcl-x<sub>s</sub>; and 2) Bcl-x<sub>s</sub> lacks an internal 63 amino domain of Bcl-x<sub>L</sub>, designated  $\Delta X_{L/s}$  (Fig. 2), we tested a series of overlapping decamer peptides spanning  $\Delta X_{L/s}$  (Table 1 and Fig. 3) for their ability to  
15 inhibit binding of Bcl-x<sub>L</sub> to cytochrome C adsorbed to wells of an ELISA plate. Data obtained over the range of 1 - 12.5  $\mu$ M are shown in Fig. 4. Observations made at higher concentrations were obfuscated by artefacts relating to differential solubility of the peptides. Peptides (7) (SEQ ID NO:1) and (8) (SEQ ID NO:8) inhibited binding by approximately 80%. Peptides (2) (SEQ ID NO:6), (3) (SEQ ID NO:7), (4) (SEQ ID NO:8), (5) (SEQ ID NO:9) and (6) (SEQ ID NO:10) inhibited binding by 30% to 40% and peptides (1) (SEQ ID NO:5), (9) (SEQ ID NO:11), (10) (SEQ ID NO:12), (11) (SEQ ID NO:13) and (12) (SEQ ID NO:14) by 5% to 20%. Thus, while the amino acid sequence (SEQ ID NO:3) common to peptide (7) (SEQ ID NO:1) and peptide (8) (SEQ ID NO:2) probably represents the dominant binding site on Bcl-x<sub>L</sub> for cytochrome C, the data point to secondary but significant  
25 roles for other subregions within  $\Delta X_{L/s}$ .  
30

Other embodiments are within the following claims.

- 51 -

What is claimed is:

1. A peptide that inhibits binding of cytochrome C to an anti-apoptotic member of the Bcl-2-related family.
2. The peptide of claim 1, wherein the anti-apoptotic member of the Bcl-2-related family is Bcl-x<sub>L</sub> or Bcl-2.  
5
3. The peptide of claim 2 wherein said peptide is a fragment of a Bcl-2-related family member.
4. The peptide of claim 3, wherein said peptide is three to seventy amino acid residues long.  
10
5. The peptide of claim 4, wherein said peptide is a fragment of Bcl-x<sub>L</sub> or Bcl-2.
6. The peptide of claim 5, wherein said peptide comprises the amino acid sequence Ala-Leu-Cys-Val-Glu (SEQ ID NO:3).  
15
7. The peptide of claim 5, wherein said peptide comprises the amino acid sequence Val-Met-Cys-Val-Glu (SEQ ID NO:4).
8. The peptide of claim 6 wherein the amino acid sequence of said peptide is SEQ ID NO:1 or SEQ ID NO:2.  
20
9. A peptide five to seventy amino acids long, said peptide comprising the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4.

- 52 -

10. A method for screening for a compound that promotes apoptosis, said method comprising:

- a) providing cytochrome C bound to a solid support;
- b) contacting the cytochrome C with a polypeptide

5 comprising the sequence of SEQ ID NO:3 or SEQ ID NO:4, in the presence of a test compound; and

- c) determining whether the test compound inhibits binding of said polypeptide to cytochrome C,

wherein inhibition of said binding is an indication that

10 said test compound promotes apoptosis.

11. The method of claim 10, wherein said polypeptide is a fusion protein comprising (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a

15 truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

12. The method of claim 10, wherein said polypeptide is (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally

20 occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

13. The method of claim 10, wherein said polypeptide is five to seventy amino acids long.

25 14. The method of claim 13, wherein said polypeptide is a fragment of Bcl-x<sub>L</sub> or Bcl-2.

- 53 -

15. A method for screening for a compound that promotes apoptosis, said method comprising:

- a) providing a polypeptide comprising the sequence of SEQ ID NO:3 or SEQ ID NO:4, the polypeptide being bound to a solid support;
- b) contacting the polypeptide with cytochrome C in the presence of a test compound; and
- c) determining whether the test compound inhibits binding of cytochrome C to the polypeptide,

wherein inhibition of said binding is an indication that the test compound promotes apoptosis.

16. The method of claim 15, wherein the polypeptide is a fusion protein comprising (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

17. The method of claim 15, wherein the polypeptide is (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

18. The method of claim 15, wherein the polypeptide is five to seventy amino acids long.

19. The method of claim 18, wherein the polypeptide is a fragment of Bcl-x<sub>L</sub> or Bcl-2.

- 54 -

20. A method for screening for a compound that inhibits apoptosis, said method comprising:

- a) providing cytochrome C bound to a solid support;
- b) contacting the cytochrome C with a polypeptide

5 comprising the sequence of SEQ ID NO:3 or SEQ ID NO:4, in the presence of a test compound; and

- c) determining whether the test compound enhances binding of said polypeptide to cytochrome C,

wherein enhancement of said binding is an indication that

10 said test compound inhibits apoptosis.

21. The method of claim 20, wherein said polypeptide is a fusion protein comprising (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a

15 truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

22. The method of claim 20, wherein said polypeptide is (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally

20 occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

23. The method of claim 20, wherein said polypeptide is five to seventy amino acids long.

25 24. The method of claim 23, wherein said polypeptide is a fragment of Bcl-x<sub>L</sub> or Bcl-2.

- 55 -

25. A method for screening for a compound that inhibits apoptosis, said method comprising:

- a) providing a polypeptide comprising the sequence of SEQ ID NO:3 or SEQ ID NO:4, the polypeptide being bound to a solid support;
  - b) contacting the polypeptide with cytochrome C in the presence of a test compound; and
  - c) determining whether the test compound enhances binding of cytochrome C to the polypeptide,
- 10 wherein enhancement of said binding is an indication that the test compound inhibits apoptosis.

26. The method of claim 25, wherein the polypeptide is a fusion protein comprising (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

27. The method of claim 25, wherein the polypeptide is (a) Bcl-x<sub>L</sub>, (b) a truncated Bcl-x<sub>L</sub> lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-x<sub>L</sub>, (c) Bcl-2, or (d) a truncated Bcl-2 lacking twenty one amino acid residues of the carboxy terminus of naturally occurring Bcl-2.

28. The method of claim 25, wherein the polypeptide is five to seventy amino acids long.

29. The method of claim 28, wherein the polypeptide is a fragment of Bcl-x<sub>L</sub> or Bcl-2.

- 56 -

30. An antibody which binds specifically to an epitope within the amino acid sequence of SEQ ID NOS:1, 2, 3 and 4.

31. The antibody of claim 30, wherein said antibody 5 is a monoclonal antibody.

32. An expression vector comprising a sequence that encodes the peptide of claim 1.

33. A method for inhibiting binding of cytochrome C to Bcl-x<sub>L</sub>, the method comprising introducing into a cell the 10 peptide of claim 1 or a peptidomimetic corresponding to the peptide of claim 1.

34. The method of claim 33, wherein the cell is in a mammal.

35. The method of claim 34, wherein the mammal is a 15 human.

36. The method of claim 35, wherein the peptide or peptidomimetic is introduced into the cell by a method comprising administering the peptide or peptidomimetic to the human.

20 37. The method of claim 35, wherein the peptide is introduced into the cell by a method comprising administering to the human an expression vector comprising a sequence that encodes the peptide.

38. The method of claim 35, wherein the human is 25 suspected of having an autoimmune disease.

- 57 -

39. The method of claim 35, wherein the human is a transplant recipient.

40. The method of claim 35, wherein the human is suspected of having cancer.

5 41. The method of claim 40, wherein the method is carried out in conjunction with chemotherapy, radiation therapy or immunotherapy.

42. A nucleic acid that encodes a polypeptide comprising a plurality of peptide domains, each of which  
10 comprises the amino acid sequence of SEQ ID NO:3.

43. A method for inhibiting release of cytochrome C into cell cytosol, the method comprising introducing into a cell the nucleic acid of claim 42.

44. The method of claim 43, wherein the cell is in a  
15 mammal.

45. The method of claim 44, wherein the mammal is a human.

46. The method of claim 45, wherein the nucleic acid is introduced into the cell by administering the nucleic  
20 acid to the human.

47. The method of claim 46, wherein the human has an immunodeficiency disease.

1/4

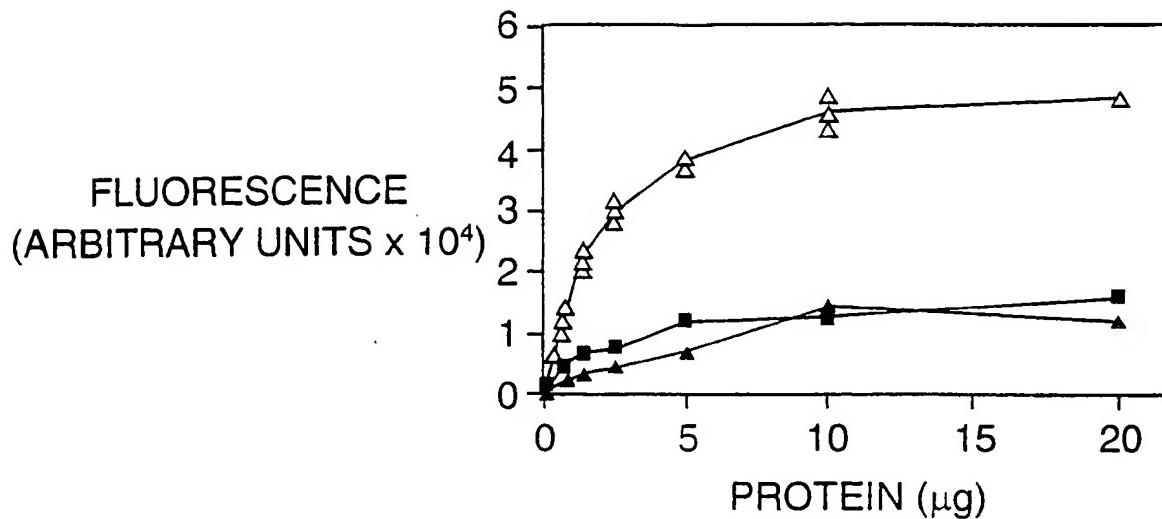


FIG. 1A

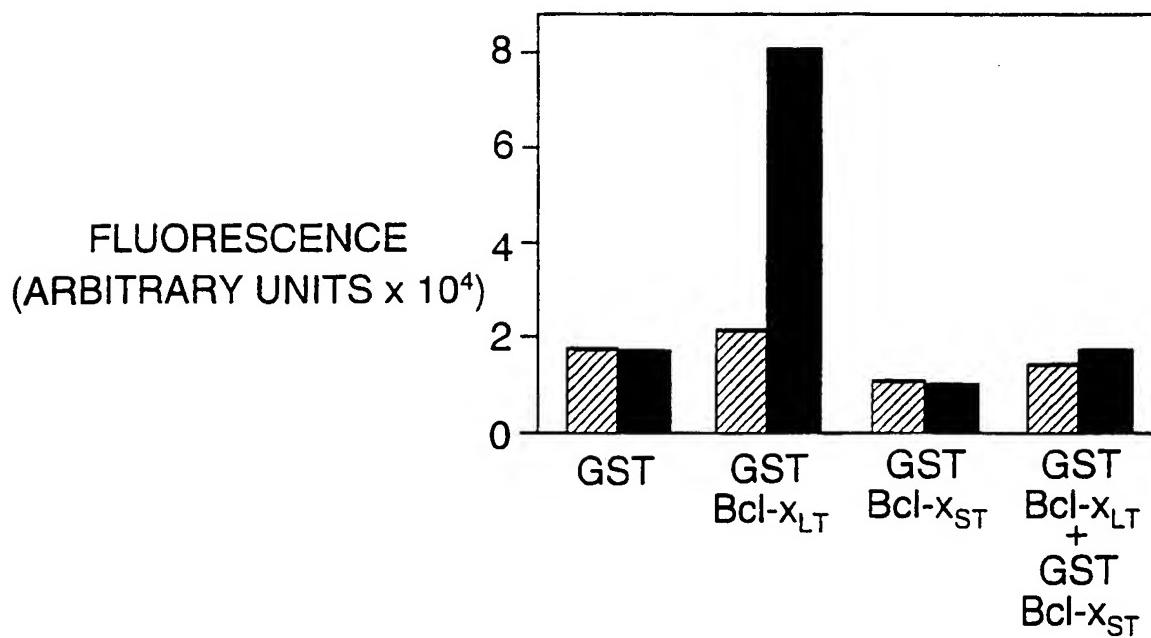


FIG. 1B

2/4

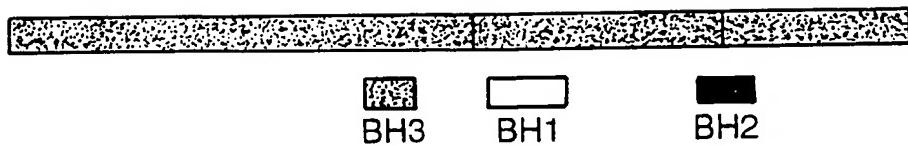
Bcl-X<sub>L</sub>Bcl-X<sub>S</sub> $\Delta X_{L/S}$ 

FIG. 2

3/4

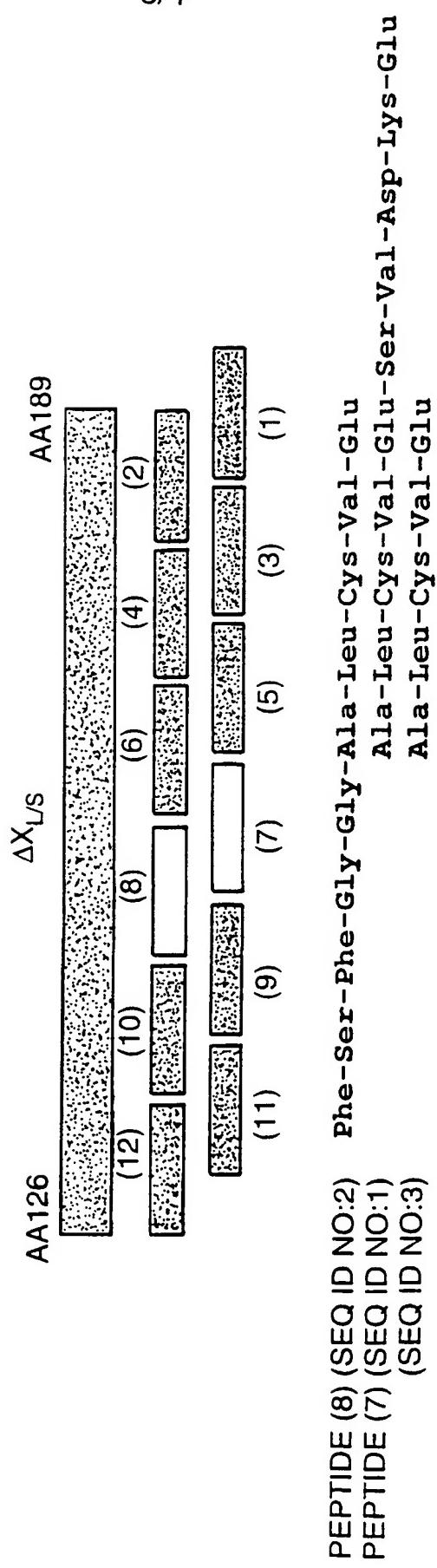


FIG. 3

4/4

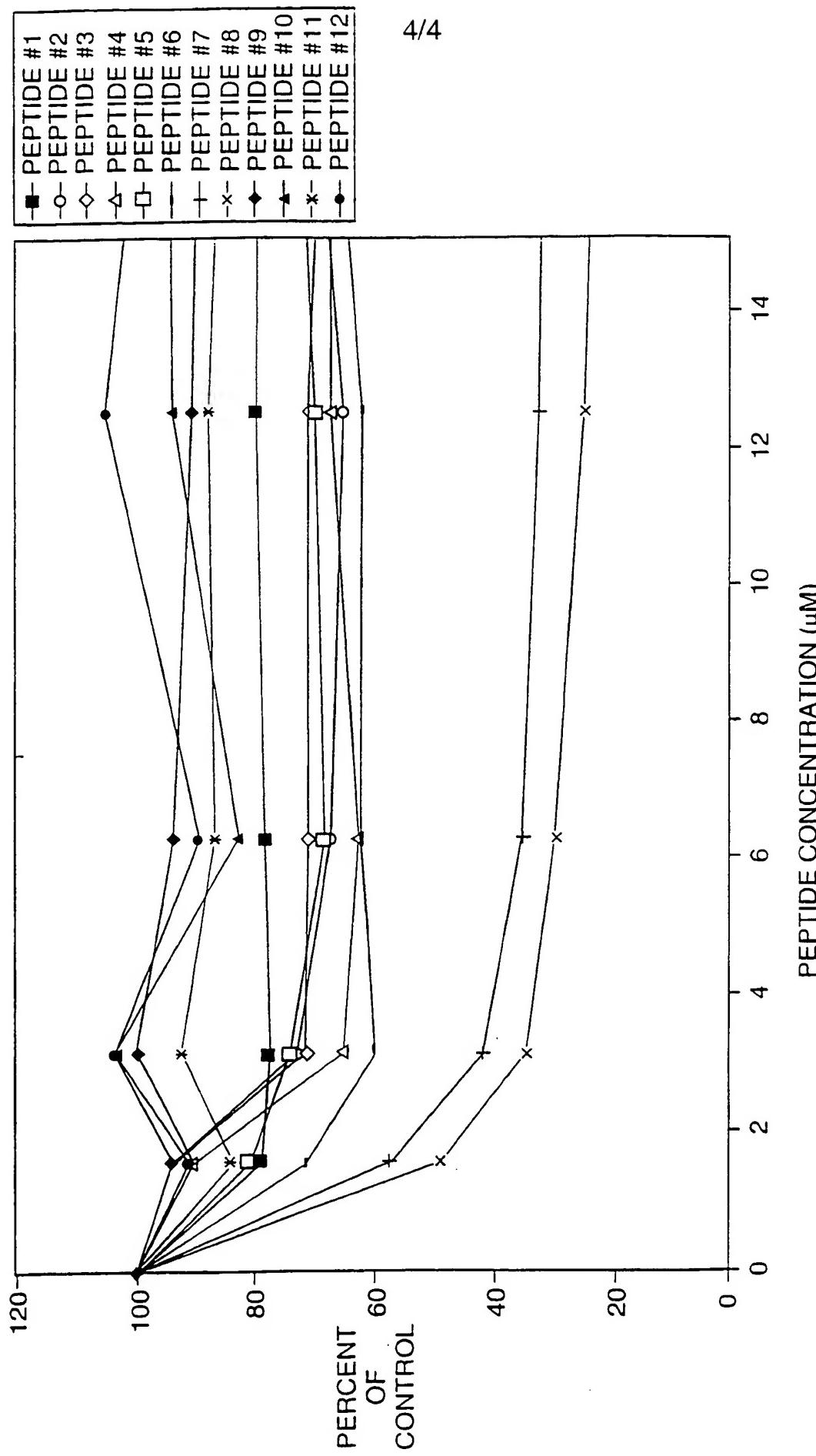


FIG. 4

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/12595

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 37/18; A61K 38/00, 38/04; G01N 33/53, 33/574, 33/48  
 US CL : 514/2, 19; 435/7.1, 7.23; 436/63; 530/330

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 19; 435/7.1, 7.23; 436/63; 530/330

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,656,725 A (CHITTENDEN et al.) 12 August 1997, columns 3-4.	1-47
Y,P	US 5,702,897 A (REED et al.) 30 December 1997, column 1.	1-47
Y,P	KHARBANDA S. et al. Role for Bcl-X <sub>L</sub> as an Inhibitor of Cytosolic Cytochrome C Accumulation in DNA Damage-Induced Apoptosis. Pro. Natl. Acad. Sci., USA. 24 June 1997, Vol. 94, pages 6939-6942, especially page 6939.	1-47

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

13 AUGUST 1998

Date of mailing of the international search report

24 SEP 1998

Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

YVONNE EYLER

Telephone No. (703) 308-0196